Original Articles

Immaturity Associated Antigens Are Lost During Induction for T Cell Lymphoblastic Leukemia: Implications for Minimal Residual Disease Detection

Mikhail Roshal,[1] Jonathan R. Fromm,[1] Stuart Winter,[2] Kimberly Dunsmore,[3] and Brent L. Wood[1]*

[1]Department of Laboratory Medicine, University of Washington Seattle, Washington

[2]Department of Pediatrics, University of New Mexico, Albuquerque, New Mexico

[3] Department of Pediatrics, Health Sciences Center, University of Virginia, Charlottesville, Virginia

Background: Induction chemotherapy for acute leukemia often leads to antigenic shifts in residual abnormal blast populations. Studies in precursor B cell ALL (B-ALL) have demonstrated that chemotherapy commonly results in the loss of antigens associated with immaturity, limiting their utility for minimal residual disease (MRD) detection. Little information is available about the stability of these antigens in precursor T cell ALL (T-ALL) though it is presumed that CD99 and terminal deoxynucleotidyl transferase (TdT) are highly informative based on limited studies.

Methods: In a longitudinal investigation, we explored patterns of lineage specific and immaturity-associated antigens in T-ALL in a large cohort of patients treated under the multicenter Children's Oncology Group (COG) protocol. All samples were analyzed using multicolor flow cytometry in a standardized fashion at a single institution.

Results: We report that markers of immaturity particularly, TdT and CD99, dramatically decline on leukemic blasts during therapy. CD34 and CD10 expression is confined to a minority of pretreatment samples and is also not stable. In contrast, lineage-associated markers including CD2, CD3, CD4, CD5, CD7, and CD8 failed to show significant trends.

Conclusions: Our study strongly argues for expansion of immunophenotyping panels for T-ALL MRD to decrease reliance on immature antigens. This study represents the first demonstration of consistent immunophenotypic shifts in T-ALL. © 2010 Clinical Cytometry Society

Key terms: cytometry; leukemia; residual; T-cell

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Detection and enumeration of minimal residual disease (MRD) is critical for risk stratification in ALL (1–3). Within the past 20 years, immunophenotyping by flow cytometry had evolved as a sensitive method to detect and enumerate residual abnormal cells (1,4–6). However, plasticity of the immunophenotype of the leukemic blasts in the setting of chemotherapy represents one major potential pitfall for their flow cytometric detection. If this plasticity is not adequately accounted for, it may compromise both qualitative and quantitative immunophenotypic evaluation. In more common acute leuke-

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*Correspondence to: Brent L. Wood, Hematopathology Laboratory, Room G7-800, 825 Eastlake Avenue E, Seattle, WA 98109, USA.

E-mail: woodbl@u.washington.edu

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mias including B cell ALL (B-ALL) (7–10) and acute myeloid leukemia (11), antigenic changes over time are well documented. In particular, prednisone induces the loss of immaturity-associated antigens CD34, CD10, and terminal deoxynucleotidyl transferase (TdT) in B-ALL blasts and acquisition of an apparently more mature immunophenotype (12,13). In contrast, changes of immunophenotype in T cell ALL (T-ALL) have not been systematically investigated, although the immunophenotype is widely presumed to be relatively stable based on limited studies (10,14).

The immunophenotype of normal T cell maturation has been previously described (15–18). Briefly, normal T-cell maturation proceeds with an orderly progression of gains and losses of multiple antigens beginning with a CD34+ T cell precursor that migrates to the thymus. Immature thymic T cells express CD99 (19), quickly gain expression of TdT, and lose CD34. During further maturation, CD10 is briefly expressed. Unlike normally maturing T cells, blasts in acute lymphoblastic leukemia (T-ALL) show maturation arrest as well as aberrant or ectopic expression of antigens that can be exploited for their identification. Because most stages of early T-cell maturation occur in the thymus, immature T cells should not be found in the peripheral blood or bone marrow, making immaturity associated antigens including TdT, CD99, CD34, and CD10 particularly attractive diagnostic targets in this disease.

TdT is a DNA polymerase that participates in nontemplate addition of nucleotides during T and B-cell receptor rearrangement. It is strongly expressed on immature cortical thymocytes (16), but is lost on normal mature T cells outside the thymus. Notably, TdT is almost uniformly expressed in precursor B (20) and T (5) cell lymphoblastic leukemias and on a subset of acute myeloid leukemia (21,22) cases. Only rare cases of TdT negative T-ALL at presentation have been reported, mostly in a setting of a very immature immunophenotype (23). Interestingly, several TdT negative MRD T-ALL cases have also been observed in small studies (24). Despite that, TdT is widely assumed to be one of the best markers for T-ALL residual disease detection.

CD99 is glycoprotein that was first isolated as an antigen that strongly reacted with T-ALL specific antibodies (25). The protein participates in T-cell adhesion (26) and is expressed widely on a variety of hematopoietic and nonhematopoietic cell types (25,27,28). Among hematopoietic elements, by far, the highest expression is observed among immature lymphoid and myeloid precursors, particularly on immature thymic T cell precursors with gradual loss of CD99 occurring upon maturation (19,25,27). Among mature normal T cells, CD99 is expressed on memory cells, but at levels significantly below the immature thymic T cells (27). It was found to be particularly useful for the detection of T-ALL in the bone marrow and peripheral blood due to virtual absence of normal thymic T cells in these specimen and expression of the antigen on a vast majority of T-ALL cases (24).

CD34 is one of the earliest markers appearing on a variety of progenitor cells, including hematopoietic progenitors. It is rapidly lost during early stages of T-cell maturation in the thymus at the stage of the early prothymocyte (18). CD34 is expressed on a minority of T-ALL, but is thought to be useful for MRD detection when present (5). During normal thymocyte development, CD10 is expressed at a somewhat later stage than CD34 (18) and its expression is brief. Although a majority of B-ALL cases express CD10, only a minority of T-ALL expresses this antigen at presentation (5,20). As with CD34, demonstration of CD10 may serve as a useful marker of T cells aberrancy when present, however, the stability of expression has not been systematically investigated.

Unlike these early markers of T-cell maturation, CD2, CD3, CD4, CD5, CD7, CD8, and CD45 are expressed on both immature and mature T cells (15–18,29,30). Mature T cells express these antigens at predictable levels and either their absence, or variations in intensity can be helpful in defining abnormal subsets and assessing T-cell maturation state. In this study, we investigated the expression and stability of immature (TdT, CD99, CD34, and CD10), lineage associated (CD2, CD3, CD4, CD5, CD7, and CD8), and CD45 antigens in a large cohort of patients undergoing chemotherapy for T-ALL.

MATERIALS AND METHODS

Patient Samples

During the period between January 2007 and May 2008, we identified 138 consecutive patients diagnosed with T-ALL and enrolled in Children's Oncology Group Protocol (COG-AALL0434) for treatment of T-ALL; of these, 74 had at least one positive sample (>0.1% abnormal blasts of mononuclear cells) on days 8, 15, or 29 after the beginning of induction chemotherapy (numbers of individual patient samples at each time point analyzed for each antigen are given in Table 2). Per COG protocol pretreatment, day-15 and day-29 samples were obtained from the bone marrow and day-8 samples were obtained from the peripheral blood. All samples were greater than 24 h of age at the time of assessment, both pretreatment and posttherapy samples, and no systematic difference in sample age between time points was noted. All patients were treated according to the clinical protocol that included cytarabine intrathecally (IT) on day 1; vincristine IV and daunorubicin hydrochloride IV on days 1, 8, 15, and 22; prednisone IV or orally twice daily on days 1–28; pegaspargase intramuscularly on days 4, 5, or 6; and methotrexate IT on days 8 and 29.

Flow Cytometry Sample Preparation and Antibodies

Blood and bone marrow samples were processed using a standard NH_4Cl whole blood lysing technique. Briefly, 100–200 μl of whole blood or bone marrow containing up to 10^6 cells were incubated with 100 μl of a titered reagent cocktail (see below) and incubated at room temperature in the dark for 15 min. About 1.5 ml

Table 1
Antibody Panels Utilized

Year of analysis	2007	2008
Tube 1	CD99 FITC, CD7 PE, CD56 PE-Cy5, CD5 PE-CY7, CD1a+CD34 APC, CD3 APC-Cy7	CD99 FITC, CD7 PE, CD56 PE-Cy5, CD34 PE-CY7, CD3 PE-TR, CD38 Alexa-594, CD1a APC, CD5 APC-Cy7, CD16 APC-A700, CD45 Pacific Blue (PB)
Tube 2	TdT FITC, CD7 PE, CD38 PerCP-Cy5.5, CD10 PE-Cy7, cytoplasmic CD3 (cCD3) APC, CD45 APC-Cy7	TdT FITC, CD7 PE, CD38 PerCP-Cy5.5, CD10 PE-Cy7, cCD3 APC, CD45 APC-Cy7
Tube 3		CD2 FITC, CD7 PE, CD8 PE-Cy5.5, CD3 PE-Cy7, CD34 PE-TR, CD4-Alexa594, CD56+CD16 Alexa647, CD5-APC-Alexa700, CD45PB

of buffered NH_4Cl containing 0.25% ultrapure formaldehyde (Polysciences) was added and incubated in the dark at room temperature for 15 min followed by a single wash with phosphate-buffered saline containing 0.3% bovine serum albumin. For samples where TdT and cytoplasmic CD3 (cCD3) were assessed, the samples were processed using the Fix-and-Perm kit (Invitrogen) per manufacturer's guidelines. All antibody reagents were obtained from either Beckman-Coulter (Fullerton, CA) or Becton-Dickinson (San Jose, CA), except for TdT, which was from SuperTechs (Rockville, MD). Slightly different antibody combinations were used in 2007 and 2008. The antibody combinations are listed in Table 1.

Samples were acquired on an optimized Becton-Dickinson LSRII monitored daily with quality control beads to validate consistent performance, and up to 750,000 events collected per sample aliquot.

Data Analysis

Flow cytometry data was analyzed with an in-house software package (Woodlist, written by B.L.W.) with appropriately applied compensation settings. To reduce confounding due to increased autofluorescence and antigen loss in dead and dying cells, low forward scatter events were excluded from analysis. T-lineage cells were identified by expression of CD7 and at least one other marker of T cell lineage in the context of appropriate forward and side-scatter parameters. Abnormal populations were identified through the detection of aberrant expression of antigens in a multiparametric manner, as appropriate for each patient and reagent combination. Use of TdT, CD99, CD10, and CD34 for exclusive gating of abnormal populations was avoided to decrease bias. CD99 expression was assessed from tube 1. CD34 expression could only be assessed in 2008 samples where CD34 was run separately from CD1a in tube 1. TdT and CD10 expression was assessed from tube 2. CD2, CD3, CD4, CD5, CD7, CD8, and CD45 values were obtained from tube 3. Enumeration of the abnormal population was verified by comparing relative frequencies of abnormal blasts across multiple tubes. To be called "positive," the sample had to have an abnormal population present in multiple tubes at similar frequencies. For follow-up samples that did not show immature markers on abnormal populations, the immunophenotype of the abnormal population had to be similar, but not necessarily identical, to the diagnostic sample in other parameters to be considered positive. Antigens common between the tubes were used for further verification of the identity of the abnormal population in each individual tube. Mean fluorescence intensity quotients (MFIQ) were obtained by dividing mean fluorescence intensity of the abnormal blasts population and normal residual T cells in individual samples. Percentage of blasts with antigen expression above normal background was determined by gating abnormal blasts expressing an individual marker at levels >95% of normal residual T cells. A sample was judged positive for an individual marker if >20% of abnormal blasts showed expression above normal background. A marker was deemed useful for quantitative MRD analysis if >50% of leukemic blasts showed expression above normal background.

Statistical Analysis

All statistical analysis was performed within SPSS16 statistical package (SPSS, Chicago, IL). Significance testing for mean fluorescence and percentage positive differences across time points was performed using paired Wilcoxon's signed rank tests. Differences in categorical variables (positive vs. negative) were performed using binomial test procedure. Significance of linear correlation between MRD percent and percent abnormal blasts showing expression of TdT, CD99, CD34, and CD10 at days 8, 15, and 29 was performed using Pearson correlation test of percent MRD within a specific time point versus percent blasts expressing an antigen at that time point.

RESULTS

As expected, the relative proportion of abnormal blasts (red) declined during treatment from day 0 (median >90%) to day 29 [median 1.1 (range, 0.1–72%)] of the total white cells. No significant linear correlation was seen between percent positive blasts within a particular time point and percent blasts expressing TdT, CD99, CD34, or CD10 at that time point (Pearson correlation p values ranging from 0.23 to 0.85).

An example of typical TdT and CD99 expression across different time points is shown in Figure 1. The normal T-cell population (shown in green) was used as a reference for establishing positive cutoffs for the antigens.

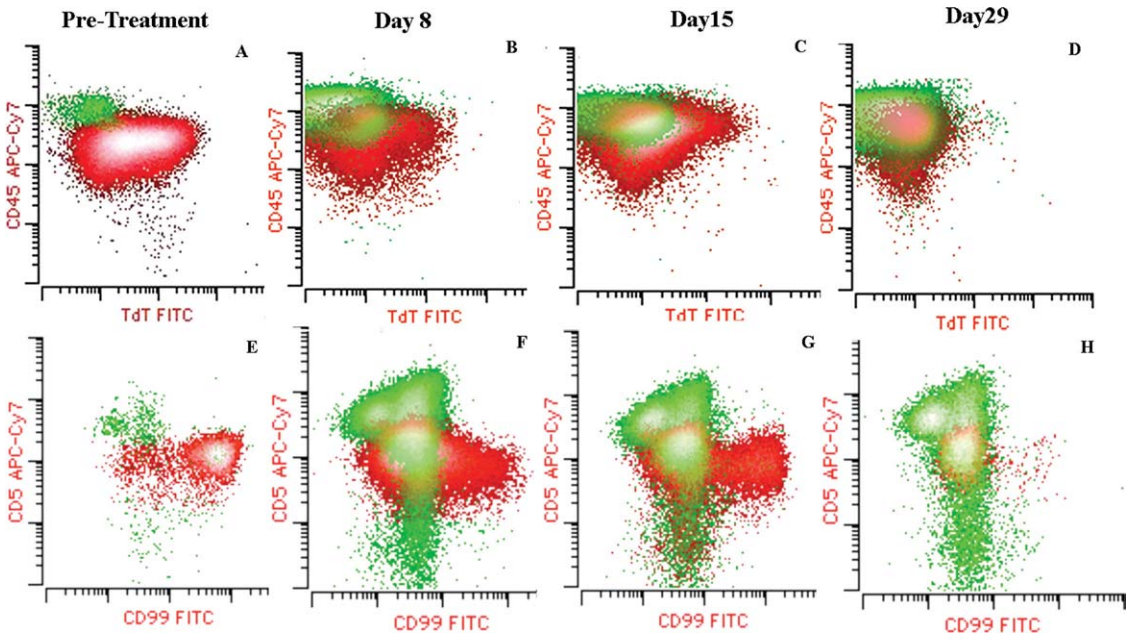

FIG. 1. Typical expression profile of CD99 (**A–D**) and TdT (**E–H**) over the 29 days postinduction chemotherapy. Normal T cell population (green) is used as an internal negative control for expression of CD99 and TdT by the abnormal T lymphoblasts (red). At day 0 (A and E), a majority of cells express CD99 and TdT. Expression of both antigens declines over time despite persistence of abnormal blast populations at days 8 (B and F), 15 (C and G), and 29 (D and H). The abnormal population is identified in each specimen by the abnormally decreased expression of CD45 and CD5 with expression of CD7 in a multiparametric assessment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Consistent with prior reports, nearly all patient samples demonstrated expression of TdT and CD99 on at least 20% of abnormal cells at diagnosis. Moreover, TdT and CD99 could be used for blast enumeration in a great majority of cases showing greater than 50% positivity for each antigen. However, expression of these antigens began to decline by day 8 and continued to decrease through day 29. Thus, only a minority of positive cases showed expression of TdT or CD99 by day 29 and in an even smaller proportion of cases could these antigens be used for blast enumeration by day 29. CD34 and CD10 were expressed on a minority of pretreatment cases and showed a similar but less dramatic decline. These results are summarized in the Figure 2 and Table 2. Overall, 59% of MRD positive cases had any of the four markers of immaturity by day 29, compared to a 100% at presentation. In the absence of immature antigens, the residual blast populations could be clearly identified in each tube using aberrant expression patterns of T-cell antigens in the respective antibody combinations, with the most frequent abnormalities being CD45 (low), CD5 (low), surface CD3 (low to absent),

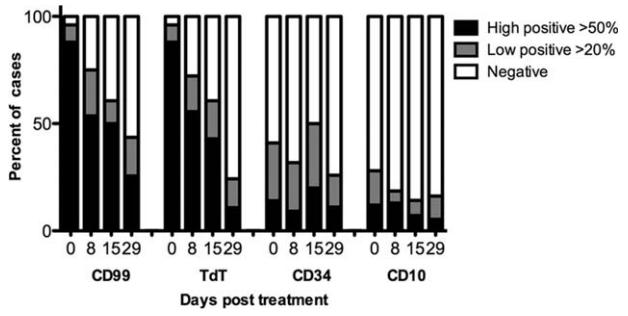

FIG. 2. Summary of the aggregate data for expression of CD99, TdT, CD34 and CD10. Cases with abnormal blasts expressing antigens at high levels (greater then 50%), shown in black and low levels (greater then 20%, but less then 50%; in gray) decline dramatically for CD99 and TdT and less so for CD34 and CD10.

Table 2
Levels of Intensity of Antigen Expression After Therapy

Time of the sample (number of individual patient samples)	% > 20%	% > 50%	MFIQ (*P* value relative to pretreatment)
Pretreatment			
CD99 (71)	96	88	1.29
TdT(71)	96	88	1.35
CD34(36)	41	14	1.07
CD10(71)	28	12	1.06
Day 8			
CD99(56)	75.0	53.6	1.24 (0.002)
TdT(54)	72.2	55.6	1.26 (<0.001)
CD34(22)	31.8	9.1	1.06 (0.615)
CD10(54)	18.6	13	1.08 (0.234)
Day 15			
CD99(28)	60.7	50	1.23 (0.034)
TdT(28)	60.7	42.9	1.20 (<0.001)
CD34(10)	50	20	1.11(0.721)
CD10(28)	14.2	7.1	1.05(0.249)
Day 29			
CD99(39)	43.6	25.6	1.14 (<0.001)
TdT(37)	24.3	10.8	1.08(<0.001)
CD34(27)	25.9	11.1	1.05 (0.091)
CD10(37)	16.2	5.4	1.07 (0.092)

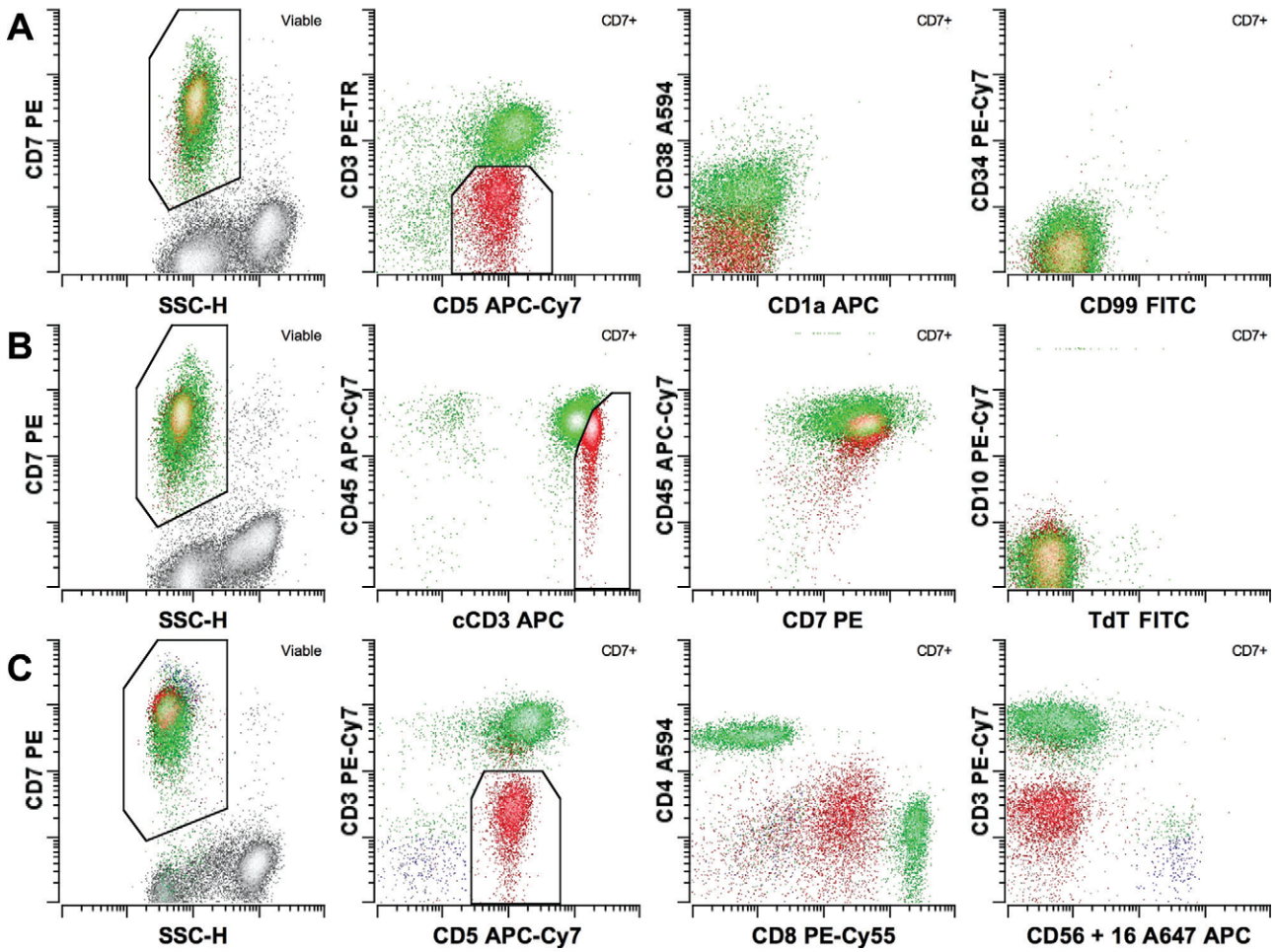

FIG. 3. A representative analysis of a day 29 sample lacking all immaturity associated antigens tested. Day 29 sample showing an abnormal immature T cell population (red) showing dim to no surface CD3, slightly low and uniform CD5, slightly high, and uniform CD7 without CD38 and lacking either CD34 or CD99 evident in tube 1 (**A**). The same population is shown to have increased expression of cytoplasmic CD3 and slightly low CD45 with absent CD10 and TdT in tube 2 (**B**). This population showed decreased expression of CD8 with low to absent CD4 and lacked NK cell-associated antigens CD56 and CD16 in tube 3 (**C**). The abnormal population represented 10.8% of the nucleated cells and has an immunophenotype the same as that seen at diagnosis except for loss of expression of CD34, CD99, and TdT. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CD7 (high), and CD38 (low or high) after exclusion of mature NK cells (CD56+, CD16+, and cCD3 negative). A representative example of an aberrant blast population from a day-29 sample lacking all the immaturity markers we tested is shown in Figure 3

Trajectory of Individual Cases

To investigate the dynamics of the antigen loss further, we analyzed the progression of CD99, TdT, CD34, and CD10 prospectively in individual cases starting with the diagnostic samples and then day 8, day 15, and day 29 follow-up samples. As expected from the aggregate data, individual cases showed gradual loss of CD99 and TdT as expressed in percentage positive leukemic blasts with mean percent loss of 28 (95% confidence interval: 19–37), 35 (20–49), and 47 (35–60) at days 8, 15, and 29 for CD99 and 30 (22–39), 44 (33–56), and 62 (51–72), respectively, for TdT. Mean fluorescence intensity for CD99 and TdT ratios paralleled the decline in the number of positive blasts. No cases gained TdT and 90% of the cases lost TdT expression as expressed as percent positive blasts or mean fluorescence intensity ratio. Only 5% of cases gained CD99 from pretreatment to day 29 with 77% losing CD99 expression in the same time period. Compared to CD99 and TdT, CD34 and CD10 were expressed at lower levels (as measured as a percentage blasts expressing a particular antigen) and on smaller number of diagnostic cases making average changes more modest. These antigens showed more mixed results with a trend toward slight declines in percent positive blasts and mean fluorescent intensities that did not reach statistical significance. The MFIQ data paralleled these findings. These results are summarized in Figure 4.

Lineage Associated Markers Remain Stable During Induction

We investigated whether the losses of TdT and CD99 are due to a global change in the antigen

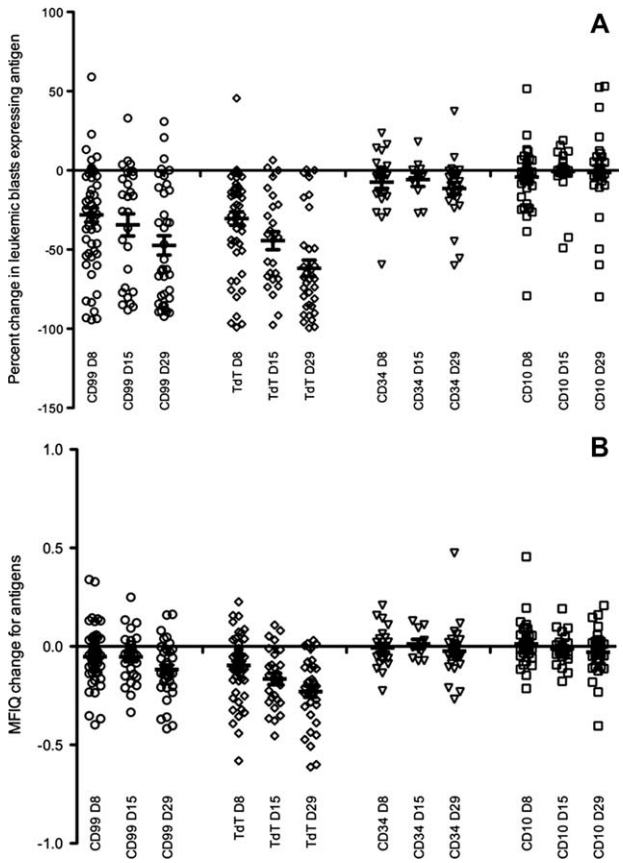

FIG. 4. Trajectory of individual cases for expression of markers of immaturity. Trajectory of individual cases shown as differences in percent expression (**A**) and mean fluorescence intensity quotient (**B**) of each marker on the abnormal blast population between the day 8, 15, and 29 samples and the pretreatment sample. Bars indicate means and standard errors of the mean for each sample group.

expression. Day 29 samples were chosen as a comparison point, because the loss of both TdT and CD99 is most pronounced at this later time point. Expression of CD2, CD3, CD4, CD5, CD7, CD8, and CD45 was analyzed for MFIQs and CD2, CD3, CD5, CD4, and CD8 for percentage expression on abnormal blasts on pretreatment and day-29 samples. Although individual cases showed minor fluctuations in individual marker expression, no systematic shift of the expression profiles was observed. With the exception of CD45, none of the markers showed a statistically significant change in the mean fluorescence intensity ($P > 0.1$) or differences in the mean number of abnormal blasts expressing the marker between pretreatment and day 29 samples. CD45 showed a slight, but statistically significant gain in mean fluorescence intensity compared to normal T cells ($P < 0.01$). Figure 5 summarizes our findings. We conclude that the expression of CD2, CD3, CD4, CD5, CD7, CD8, and CD45 is relatively stable over time with no systematic shift evident between pretreatment and day-29 samples.

DISCUSSION

This study establishes that similar to B-ALL and AML, the immunophenotype of T-ALL is labile in a setting of multidrug chemotherapy. The decline of the expression of markers of immaturity on the leukemic blasts does not appear to be a function of residual disease burden as no correlation between the number of leukemic blasts and TdT, CD99, CD34, or CD10 expression on these blasts was found at any time point. On the other hand, the loss of these antigens may indicate relatively worse response to therapy, because we only analyzed samples that were positive for MRD during induction chemotherapy. These findings parallel those of B-ALL that demonstrated apparent partial immunophenotypic maturation of leukemic blasts (9). In that disease,

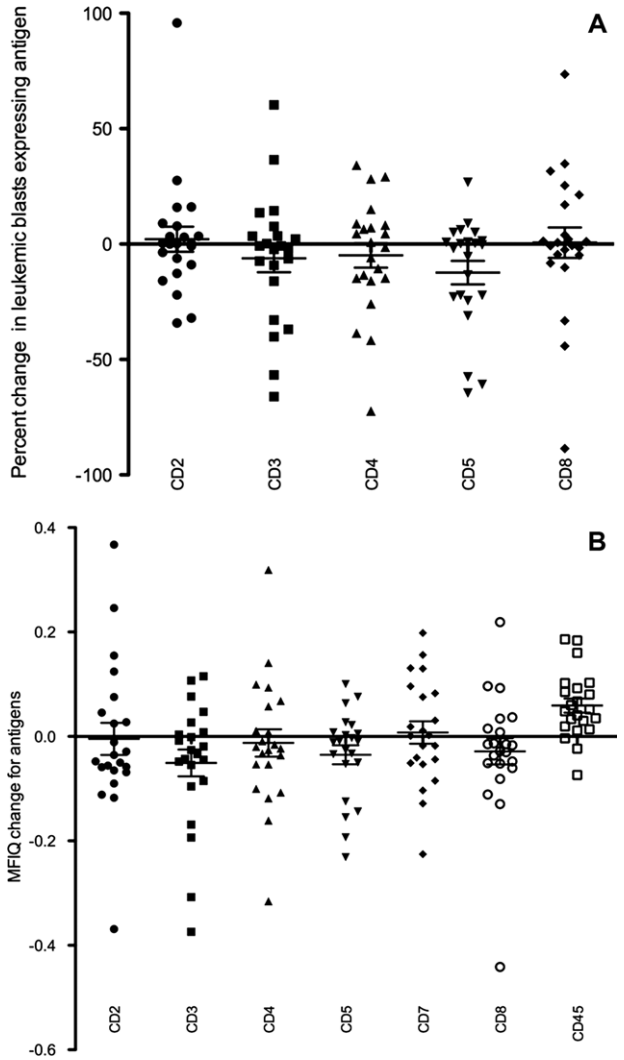

FIG. 5. Trajectory of individual cases for expression of markers of T cell lineage associate markers and CD45. Trajectory of individual cases shown as differences in percent expression (**A**) and mean fluorescence intensity quotient (**B**) of each marker on the abnormal blast population between day 29 samples and pretreatment samples. Error bars indicate standard errors of the mean for each sample group.

prednisone appears to be responsible for the immunophenotypic changes involving CD34 and CD10 (12). One recent publication also demonstrated the specific downregulation of TdT gene expression on B-ALL by prednisone (13). Interestingly, steroids were part of the chemotherapy for the patients in this study and could be responsible for the observed immunophenotypic changes. The biological pathways that lead to the dramatic downshift in immaturity associated antigens during induction chemotherapy in T-ALL remains unexplored and could provide a valuable insight into the nature of chemotherapy resistant disease. It is not clear if the loss of TdT and CD99 represent the selective survival of an antigen negative subpopulation or downregulation on the previously positive blasts that survive chemotherapy.

Although it is highly likely that exposure to chemotherapic agents is responsible for the differences in antigen expression observed between samples obtained at various time points, we are unable to separate the contributions of in vivo versus ex vivo exposures. All the samples from various time points were collected and processed in a relatively uniform manner, and no consistent differences in sample viability were noted despite some variability in the time between sample collection and analysis, arguing against sample degradation as the likely explanation for the changes. It is possible that less dramatic changes in the loss of immature antigens might be seen on samples tested closer to the time of collection, but these samples are typical of those obtained in multi-institutional clinical trials using centralized testing and the need to reliably detect residual disease in samples of this type remains. This data suggest that the reliance on immature antigens in this context is to be avoided, particularly when other more reliable targets exist.

Based on the antigens we analyzed, loss of markers of immaturity does not appear to be a part of a consistent shift toward either a more mature or less mature antigen profile, as aside from immature antigens, the leukemic blasts had a relatively stable antigen expression profiles between pretreatment and day-29 samples. However, more work is required to elucidate the mechanism responsible for downregulation of the markers of immaturity in T-ALL and potential changes in maturational state.

The study highlights that two of the antigens that are reputed to be the most reliable in detection of T-ALL, TdT, and CD99 appear to have inconsistent utility in the setting of MRD detection during induction chemotherapy. Our conclusion stands is contrast to previous smaller studies of TdT (5) and CD99 (24), which suggested their utility in the setting of MRD detection. Although the reasons for the discrepancy are not absolutely clear, we suggest that several factors could be responsible. First, both studies contained either no (24) or a small number (5) of day-29 samples where the losses of both TdT and CD99 are most pronounced. Second, it is possible that the use of limited (three to four color) antibody panels in the earlier studies led to an underestimation of the total minimal disease burden by relying heavily on CD99 and/or TdT expression.

This study highlights the need for caution when using immaturity markers for MRD evaluation even when the prior immunophenotype is known. Specifically, MRD may still be present in the absence of conventional immaturity markers. In our study, 41% of samples positive for MRD at day 29 lacked any of the immaturity markers tested. In contrast to the markers of immaturity, lineage-associated antigens do not show major directional shifts and mostly show minor fluctuations in one or two, making them valuable and informative components of expanded immunophenotyping panels for MRD detection. In the cases that lacked antigens of immaturity, preservation of mature marker profiles allowed for confident MRD determination. It is our experience that expansion of immunophenotyping panels to allow a higher level multiparametric analysis decreases reliance on individual antigens, particularly markers of immaturity, and leads to greater confidence and accuracy in identifying abnormal populations in T-ALL. Our finding should facilitate optimization of T-ALL detection-specific antibody panels.

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